

## Effect of Transforming Growth Factor- $\beta$ 1 on the Cell Growth and Epstein–Barr Virus Reactivation in EBV-Infected Epithelial Cell Lines

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Transforming growth factor (TGF)- $\beta$ 1 is a multifunctional cytokine that plays important roles in regulating cell growth and differentiation in many biological systems. In this study, we found that gastric tissue-derived Epstein–Barr virus (EBV)-infected epithelial cell lines GT38 and GT39 had resistance to TGF- $\beta$ 1-mediated growth inhibition and apoptosis compared to a TGF- $\beta$ 1-susceptible gastric carcinoma cell line HSC-39. However, TGF- $\beta$ 1 partially induced EBV reactivation in GT38 and GT39 cells, as shown by the induction of EBV immediate-early BZLF1 RNA and its protein product ZEBRA and early antigen-D. The expressions of TGF- $\beta$  receptor I and II were detected in GT38 and GT39 cells by Northern and Western blot analyses. Both cell lines spontaneously produced the TGF- $\beta$ 1, which was sufficient for inhibiting cell growth of HSC-39 cells. Taken together, these data suggest that TGF- $\beta$ 1 may be a key factor for EBV reactivation and selective growth of EBV-infected epithelial cells *in vivo*. © 2001 Academic Press

### INTRODUCTION

Epstein–Barr virus (EBV) ubiquitously infects the majority of humans and is the causative agent of infectious mononucleosis. EBV infection has been closely linked to various epithelioid and lymphoid malignancies (Rickinson and Kieff, 1996). Many studies have also indicated a relationship between EBV and gastric carcinoma (Shibata and Weiss, 1992; Fukayama *et al.*, 1994; Sugiura *et al.*, 1996). Analysis of the EBV DNA, which shows clonality from gastric carcinoma cells, indicated that tumors arose from a single EBV-infected cell, thus suggesting that EBV infection had occurred in the very early stage of tumor development (Imai *et al.*, 1994). Furthermore, elevated EBV antibody titers to viral lytic antigens in patients' sera with EBV-associated gastric adenocarcinoma presumably reflected EBV reactivation in the gastric carcinoma cells (Imai *et al.*, 1994; Levine *et al.*, 1995). Little, however, is known about the mechanisms for clonal expansion of EBV-infected gastric carcinoma cells and reactivation of viral latency in the EBV-positive epithelial cells.

Transforming growth factor (TGF)- $\beta$  superfamily members, including activin, bone morphogenetic protein, and TGF- $\beta$ , play important regulatory roles in cell growth,

morphogenesis, cell differentiation, and apoptosis (Sporn and Roberts, 1992; Massague, 1998). The effects of TGF- $\beta$  on cell growth *in vitro* can be positive or negative depending on cell types, culture conditions, and presence of other growth factors. In most cells of epithelial origin, TGF- $\beta$  is a potent growth inhibitor (Sporn and Roberts, 1992).

Gastric carcinoma cells express a number of growth factors, gastrointestinal hormones, and cytokines that may enhance the growth of these tumor cells through potential autocrine, paracrine, and juxtacrine pathways (Tahara *et al.*, 1990). Among them, elevated levels of TGF- $\beta$  have been reported in patients with human gastric cancer (Mizoi *et al.*, 1993; Naef *et al.*, 1997).

In the relationship of TGF- $\beta$  and EBV, TGF- $\beta$  induces viral reactivation in EBV-infected B cells (di Renzo *et al.*, 1994). Besides, EBV abrogates the TGF- $\beta$ -mediated growth inhibition in human B cells (Arvanitakis *et al.*, 1995). These observations lead us to postulate that TGF- $\beta$  may be an important factor to induce certain virological and immunological findings in EBV-associated gastric carcinoma. We hypothesize that TGF- $\beta$  may control the growth and EBV reactivation of EBV-infected epithelial cells. Although the interaction between EBV and lymphoid cells has been studied extensively, *in vitro* studies of EBV in epithelial cells has been hampered by the lack of an experimental cell system, which is susceptible to EBV. To explore our hypothesis, we examined the effect of TGF- $\beta$ 1 on EBV-infected epithelial cell lines

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GT38 and GT39 derived from gastric tissues (Tajima *et al.*, 1998). These cell lines were established from non-cancerous portions of the gastric carcinoma of two patients and had spontaneous EBV reactivation in small numbers of cells. The EBV latency was referred to as type III, which expresses EBNA1, EBNA2, and LMP1 (Takasaka *et al.*, 1998; Murakami *et al.*, 2000). These cell lines formed colonies in soft agar (Kanamori *et al.*, 2000) and tumors in SCID mice (Murakami *et al.*, 2000).

In this study, we found that GT38 and GT39 cell lines resisted TGF- $\beta$ 1-mediated growth inhibition and apoptosis, while latent EBV in the cells was partially reactivated by TGF- $\beta$ 1. We analyzed the expression of TGF- $\beta$  receptors (Rs) and TGF- $\beta$ 1-mediated signals in these cell lines and found that these cell lines spontaneously produced TGF- $\beta$ 1, which may modulate the cell growth and EBV reactivation.

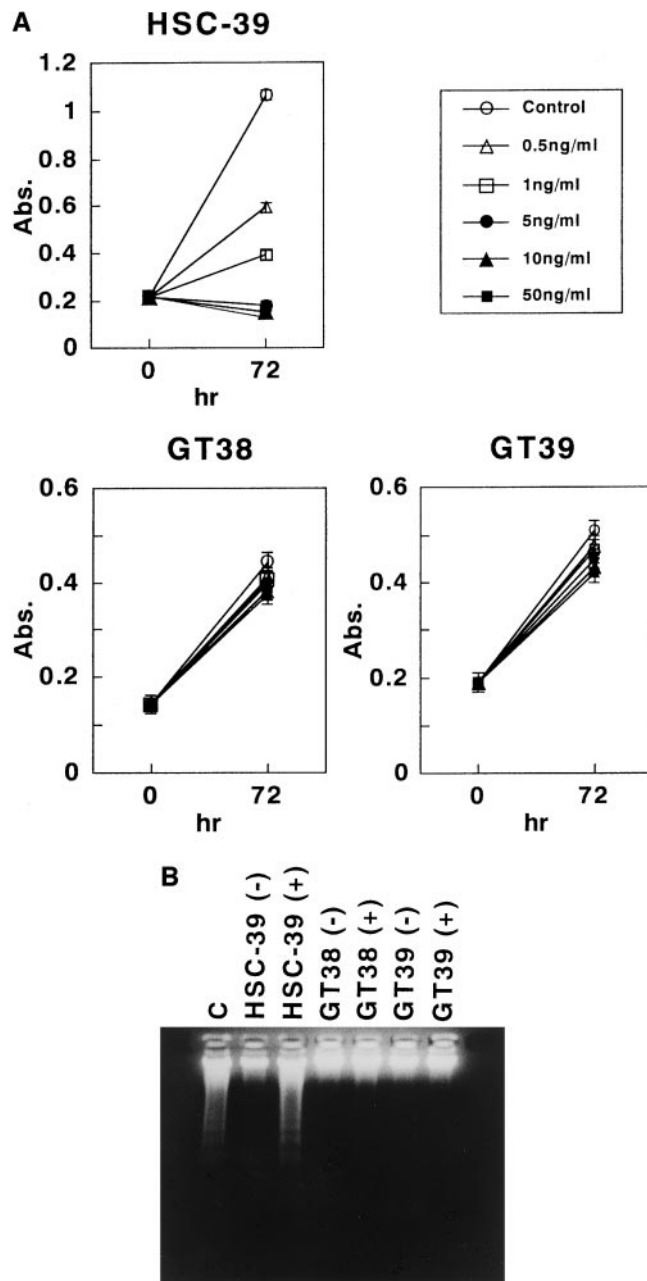
## RESULTS

### Effect of TGF- $\beta$ 1 on the cell growth and apoptosis

EBV-positive and -negative B cells exhibit a differential response to TGF- $\beta$ . EBV-negative B cells are sensitive to TGF- $\beta$ -mediated growth inhibition, whereas EBV-positive B cells lose that sensitivity (Arvanitakis *et al.*, 1995). To characterize the effect of TGF- $\beta$ 1 on growth of GT38 and GT39 cells, we measured the TGF- $\beta$ 1-mediated growth inhibition in parallel to HSC-39 cells in which growth inhibition and apoptosis were induced by TGF- $\beta$ 1 (Yanagihara and Tsumuraya, 1992). By the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay, we observed differential sensitivity to TGF- $\beta$ 1 in growth of these cell lines (Fig. 1A). Growth of HSC-39 cells was inhibited with TGF- $\beta$ 1 in a dose-dependent manner. It was inhibited at 0.5 ng/ml of TGF- $\beta$ 1 partly and almost completely at 5 ng/ml or higher concentrations. On the other hand, the growth of GT38 and GT39 cells was not or little inhibited at those concentrations of TGF- $\beta$ 1. We analyzed whether TGF- $\beta$ 1 induces apoptosis in these cell lines by a DNA fragmentation test (Fig. 1B). DNA fragmentation was induced in HSC-39 cells by TGF- $\beta$ 1. In contrast, DNA fragmentation was not observed in GT38 and GT39 cells.

### Expression of TGF- $\beta$ Rs I and II

Lack of expression of TGF- $\beta$  Rs has been reported in gastric cancer cell lines (Park *et al.*, 1994; Yamamoto *et al.*, 1996). We investigated the expression of TGF- $\beta$  Rs I and II in GT38, GT39, a gastric cancer cell line MKN1, and HSC-39 cells by Northern and Western blot analyses. Expression of mRNAs of TGF- $\beta$  Rs I (5.2 kb) and II (5.5 kb) were detected in all cell lines to similar levels (Fig. 2A). Expression of proteins of TGF- $\beta$  Rs I and II were also detected in all cell lines (Fig. 2B).



**FIG. 1.** Effect of TGF- $\beta$ 1 on the cell growth and apoptosis in HSC-39, GT38, and GT39 cells. (A) Cells were seeded at  $5 \times 10^3$  cells/100  $\mu$ l per well and incubated for 24 h and then were cultured for 72 h with various concentrations of TGF- $\beta$ 1. Cell growth was measured by MTT assay. Results are the means and standard deviations for five experiments. (B) Cells ( $1 \times 10^6$  cells/ml) were cultured with (+) or without (-) TGF- $\beta$ 1 (10 ng/ml) for 24 h at 37°C. DNA was isolated from the cells, subjected to the agarose gel electrophoresis, and visualized with ethidium bromide. Anti-IgM-treated Ramos cells were used as the positive control for the DNA fragmentation (lane C).

### Effect of TGF- $\beta$ 1 on EBV reactivation

TGF- $\beta$ 1 induces EBV reactivation in B cell lines (di Renzo *et al.*, 1994). We studied the effect of TGF- $\beta$ 1 on EBV reactivation in GT38 and GT39 cells (Fig. 3). TGF- $\beta$ 1 induced the EBV immediate-early gene BZLF1, protein

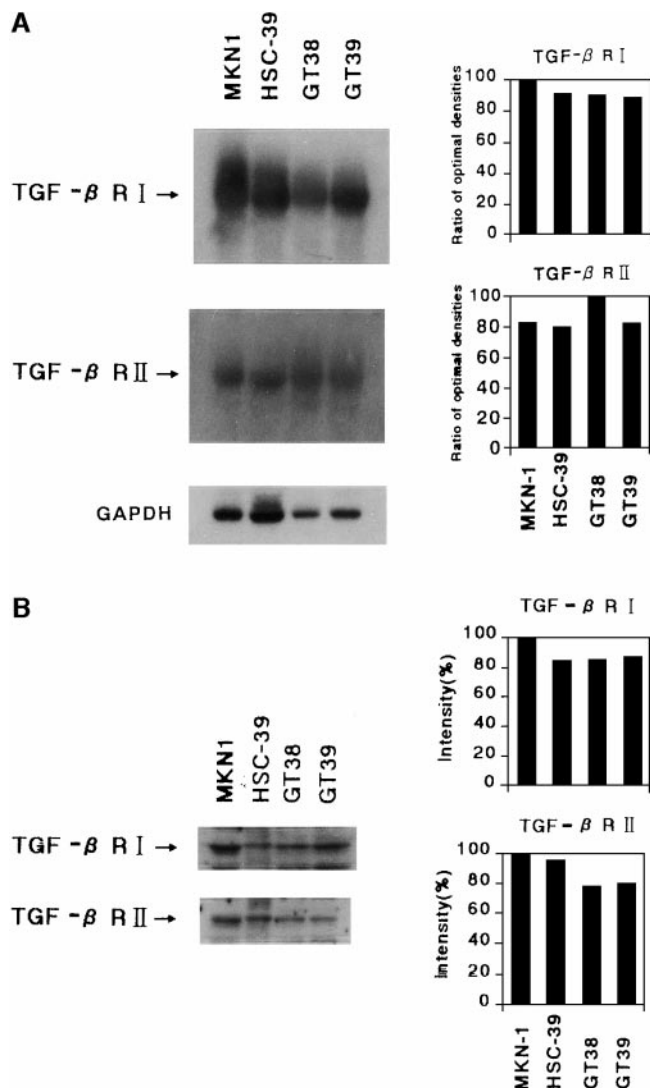


FIG. 2. Expression of TGF- $\beta$  Rs I and II in GT38, GT39, MKN1, and HSC-39 cells. (A) Thirty micrograms of total RNA was subjected to the Northern blotting. GAPDH mRNA was hybridized to show that comparable amounts of mRNA had been loaded in each lane (left). The autoradiographs for TGF- $\beta$  R I, II, and GAPDH mRNAs were analyzed by laser densitometry and the RNA levels of TGF- $\beta$  Rs were normalized to the corresponding GAPDH signals (right). (B) Thirty micrograms of protein from the cells was separated electrophoretically, blotted onto membranes, and then immunoreacted with anti-human TGF- $\beta$  Rs I and II Abs. The positions of the TGF- $\beta$  Rs I (53 kDa) and II (70 kDa) bands are indicated by an arrow (left). The autoradiographs for TGF- $\beta$  Rs I and II band intensities were analyzed by laser densitometry (right).

ZEBRA (Countryman *et al.*, 1987; Kenney *et al.*, 1989). We analyzed the time course of the TGF- $\beta$ 1-dependent induction of BZLF1 mRNA, ZEBRA, and EA-D, markers of EBV reactivation in GT38 cells (Fig. 3A). The levels of BZLF1 and BRLF1 expression increased at 8 h and reached a maximal level at 48 h. The levels of ZEBRA expression increased at 24 h and reached a maximal level at 48 h. The size of ZEBRA in Akata cells is smaller than that of prototypic virus strain B95-8 (Packham *et al.*, 1993) and than those of GT38 and GT39 cells (Takasaka

*et al.*, 1998). The levels of EA-D expression also increased by 48 h and reached a higher level at 72 h. We tested the effective doses of TGF- $\beta$ 1 for the EBV reactivation in GT38 cells (Fig. 3B). ZEBRA and EA-D were induced at 5 ng/ml and greater concentrations of TGF- $\beta$ 1. ZEBRA and EA-D inductions were observed similarly in GT39 (data not shown) cells.

#### TGF- $\beta$ 1 Rs of GT38 cells mediate TGF- $\beta$ 1 signals

To characterize TGF- $\beta$ 1-mediated signal transduction in GT38 cells, we tested for expression of plasminogen activator inhibitor-1 (PAI-1) and junB, which are up-regu-

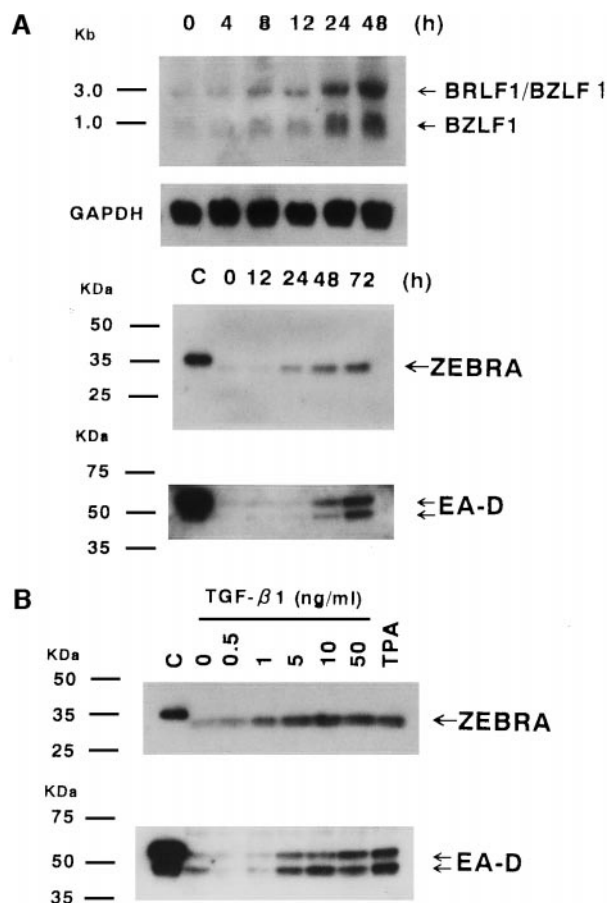
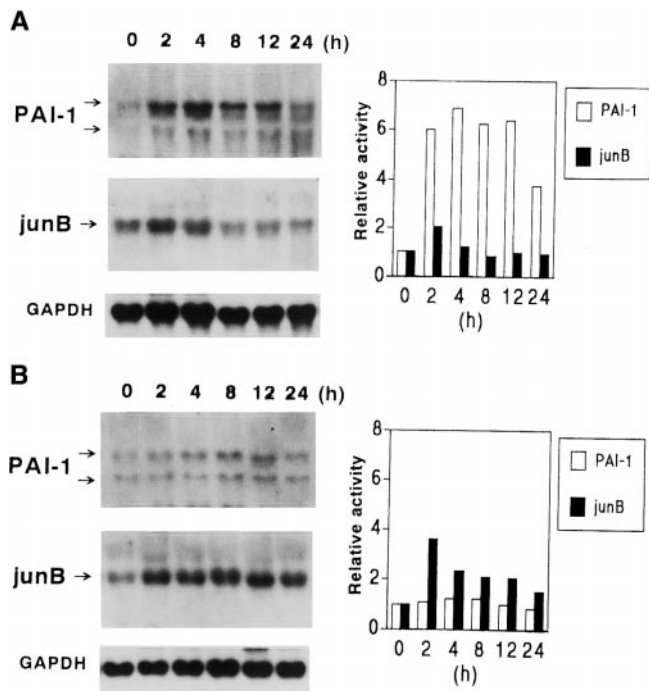


FIG. 3. Effect of TGF- $\beta$ 1 on EBV reactivation in GT38 cells. (A) Time course for EBV reactivation in TGF- $\beta$ 1-treated GT38 cells. GT38 cells were cultured with or without TGF- $\beta$ 1 (10 ng/ml). Total RNA was extracted from GT38 cells at 0, 4, 8, 12, 24, and 48 h after treatment with TGF- $\beta$ 1. Northern hybridization was performed using cDNA probes for BZLF1 and GAPDH. The expression of ZEBRA and EA-D was analyzed by Western blot analysis. Forty micrograms of protein from the cells at the indicated times after treatment with TGF- $\beta$ 1 (10 ng/ml) was separated electrophoretically, blotted onto membranes, and then immunoreacted with anti-ZEBRA and anti-EA-D Abs. Anti-IgG-treated Akata cells (lane C) and 12-O-tetradecanoylphorbol-13-acetate-treated GT38 cells for 72 h were used as the positive controls for ZEBRA and EA-D, respectively. (B) Dose response of TGF- $\beta$ 1 on EBV reactivation. GT38 cells were treated with various concentrations of TGF- $\beta$ 1 for 72 h. ZEBRA and EA-D were detected in the cells by immunoblotting with anti-ZEBRA and anti-EA-D Abs as shown in (A).



**FIG. 4.** TGF- $\beta$ 1 induction of PAI-1 and junB expression in HSC-39 (A) and GT38 (B) cells. Cells were cultured with fresh medium for 24 h at 37°C and then exposed to TGF- $\beta$ 1 (10 ng/ml). The cells were harvested at various times. Northern hybridization was performed on the same membrane using cDNA probes for PAI-1, junB, and GAPDH, respectively (left). Activation (fold induction) of the PAI-1 and junB expressions in TGF- $\beta$ 1-treated vs the control cells was evaluated with laser densitometry (right). Ratios of the optical densities between PAI-1/junB and the corresponding GAPDH signals were calculated and the RNA levels of PAI-1/junB were normalized.

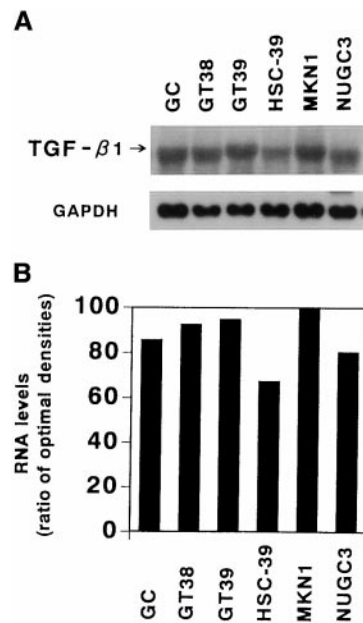
lated by TGF- $\beta$ 1 (Denler *et al.*, 1998; Jonk *et al.*, 1998). TGF- $\beta$ 1 rapidly induced PAI-1 and junB expression more than sixfold and twofold, respectively, in HSC-39 cells to the untreated cells (Fig. 4A). In contrast, TGF- $\beta$ 1 induced little PAI-1 expression; however, JunB was induced rapidly and strongly in GT38 cells (Fig. 4B).

#### GT38 and GT39 cells express TGF- $\beta$ 1 mRNA

TGF- $\beta$ s are overexpressed in gastric carcinoma tissues and derived cell lines (Mizoi *et al.*, 1993; Naef *et al.*, 1997; Yamamoto *et al.*, 1996). We examined expression of TGF- $\beta$ 1 mRNA by Northern blot analysis in GT38, GT39, HSC-39, in additional gastric cancer cell lines MKN1 and NUGC3, and in an EBV-positive gastric carcinoma specimen (GC). TGF- $\beta$ 1 mRNA with about 2.5 kb was detected in the all cell lines and the gastric carcinoma tissue (Fig. 5A). The TGF- $\beta$ 1 mRNA levels of GT38, GT39, and MKN1 cells were slightly greater than in the other cell lines and the carcinoma tissue (Fig. 5B).

#### GT38 and GT39 cells release biologically active TGF- $\beta$ 1

We analyzed whether GT38 and GT39 cells produce TGF- $\beta$ 1 in the supernatants using the fluorometric sand-



**FIG. 5.** Expression of the TGF- $\beta$ 1 mRNA in a gastric carcinoma tissue (GC), GT38, GT39, HSC-39, MKN1, and NUGC3 cells. (A) Thirty micrograms of total RNA from the cells was subjected to Northern blotting for TGF- $\beta$ 1. GAPDH was used as the internal control. (B) The autoradiograph (A) was analyzed with a laser densitometry. Ratios of the optical densities between TGF- $\beta$ 1 and the corresponding GAPDH signals were calculated and the RNA levels of TGF- $\beta$ 1 were normalized.

wich ELISA and the growth inhibition assay. These tests enable TGF- $\beta$ 1 to be characterized as an active form on direct assay of the supernatant, or as both latent and active forms (total TGF- $\beta$ 1), following heat treatment for 10 min at 80°C (Brown *et al.*, 1990). Heat treatment releases the TGF- $\beta$ 1 from a carrier protein.

The active and total TGF- $\beta$ 1 were assayed in supernatants from GT38 and GT39 cells by ELISA (Table 1). The TGF- $\beta$ 1 in supernatants from GT38 and GT39 cells were detected with similar levels.

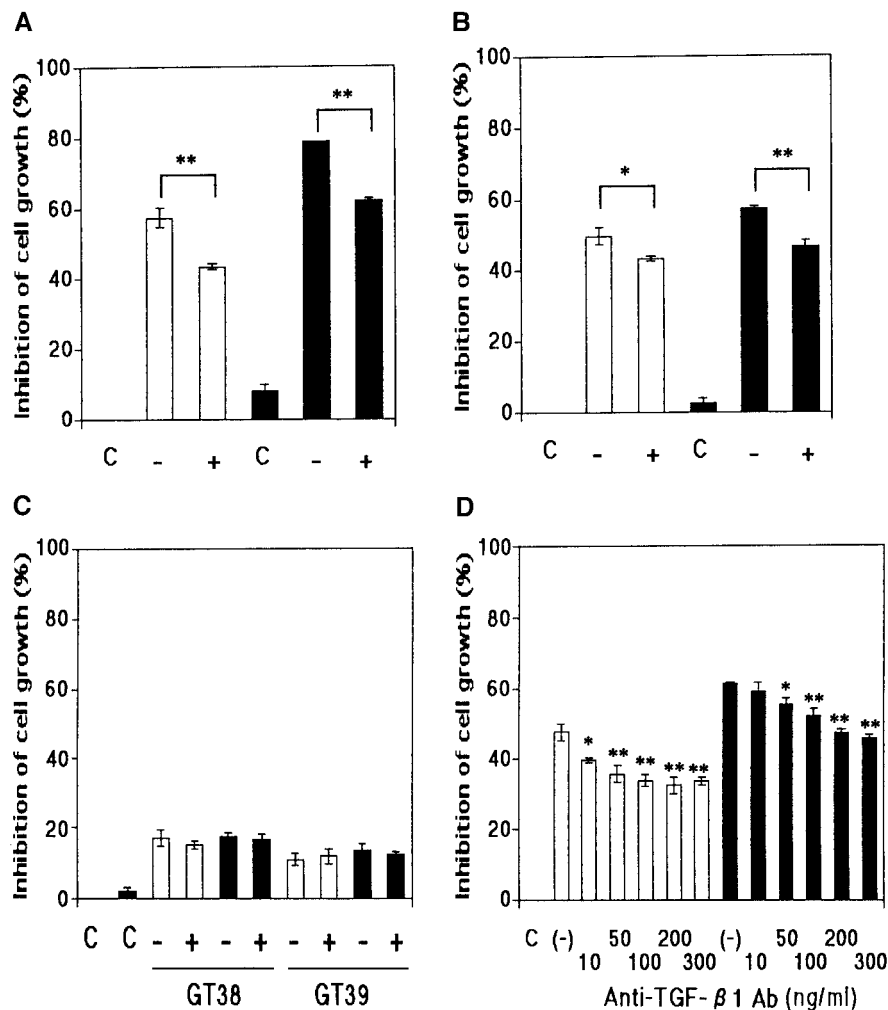
The biological activity of TGF- $\beta$ 1 was examined by a growth inhibition assay using the HSC-39 cells, a sensitive cell line to TGF- $\beta$ 1. As expected, the supernatants from GT38 or GT39 cells inhibited the growth of HSC-39 cells (Figs. 6A and 6B). We demonstrated that the growth

**TABLE 1**  
Amount of TGF- $\beta$ 1 in the Culture Supernatants from GT38 and GT39 Cells

Cells	Active (ng/ml)	Total (ng/ml)
GT38	0.37 $\pm$ 0.09	0.62 $\pm$ 0.12
GT39	0.32 $\pm$ 0.05	0.54 $\pm$ 0.15

*Note.* The cells ( $1 \times 10^5$  cells/ml) were cultured for 24 h, and the amounts of active and total TGF- $\beta$ 1 were assayed in the culture supernatants by ELISA. Values are expressed as the mean  $\pm$  standard deviation. Data represent the means of five experiments.





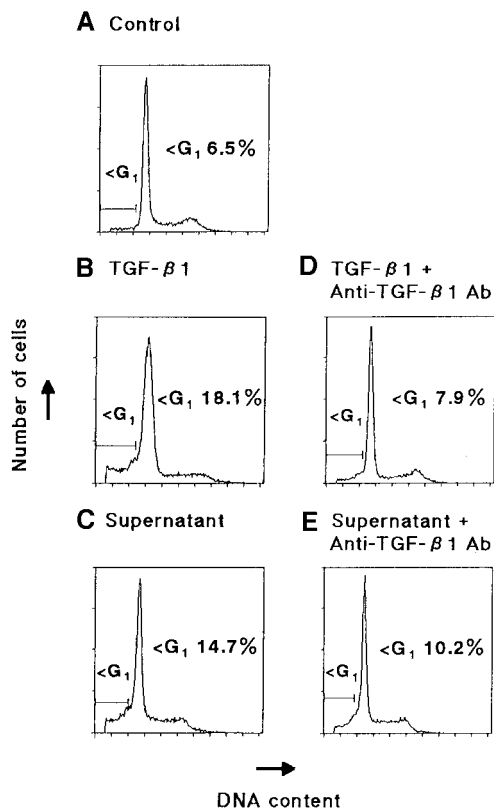
**FIG. 6.** Detection of TGF- $\beta$ 1 activity in the supernatants from GT38 or GT39 cells. TGF- $\beta$ 1 activity was assessed by the growth inhibition using the HSC-39 cells. HSC-39 ( $5 \times 10^3$  cells/100  $\mu$ l) cells were cultured in the supernatants from GT38 (A) or GT39 (B) cells for 72 h. The supernatants were untreated (open bar) or treated at 80°C for 10 min (shaded bar). The supernatants were treated with (+) or without (-) anti-TGF- $\beta$ 1 Ab (100 ng/ml). (C) Effect of the supernatants from GT38 or GT39 cells on the cell growth of GT38 cells. The cells were cultured and growth was determined as shown in (A) and (B). (D) The dose response of anti-TGF- $\beta$ 1 Ab on the blocking the growth-inhibitory activity of the supernatant from GT38 cells to HSC-39 cells. The cells were cultured and the growth was determined as shown in (A) and (B). Results are the means and standard deviations for five experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$  compared with the untreated control group analyzed by the unpaired  $t$  test.

inhibition was due to active TGF- $\beta$ 1 in the supernatants by blocking the growth inhibition by anti-TGF- $\beta$ 1 antibody (Ab). The growth-inhibitory activity in the supernatants was reduced partially by the anti-TGF- $\beta$ 1 Ab. The growth-inhibitory activity of heated supernatants was observed at more higher levels than those of the unheated supernatants and they were reduced similarly with anti-TGF- $\beta$ 1 Ab. The supernatants from GT38 or GT39 cells slightly inhibited the growth of GT38 cells; however, the growth inhibition with the fresh and heated supernatants was not reduced with anti-TGF- $\beta$ 1 Ab (Fig. 6C). To confirm the growth inhibition by TGF- $\beta$ 1, we analyzed in more detail the effect by the anti-TGF- $\beta$ 1 Ab in the GT38 supernatant. Anti-TGF- $\beta$ 1 Ab blocked the growth-inhibition in a dose-

dependent fashion (Fig. 6D). The Ab also blocked the growth inhibition of the heated supernatant.

We further analyzed whether the supernatant from GT38 cells induces apoptosis in HSC-39 cells by flow cytometry (Fig. 7). Apoptotic cells contain less DNA, which is due to DNA fragmentation than the cells in  $G_0/G_1$  phase (Fukuda *et al.*, 1995). The proportion ( $<G_1$ ) of apoptotic cells was 6.5% in the control HSC-39 cells. The proportion increased in the cells incubated with TGF- $\beta$ 1 or with supernatant from GT38 cells to 18.1 or 14.7%, respectively. The proportions reduced to 7.9 or 10.2%, respectively, in the cells cultured with TGF- $\beta$ 1 or the supernatant which was pretreated by anti-TGF- $\beta$ 1 Ab.

We also observed that the supernatants from GT38



**FIG. 7.** Supernatant from GT38 cells induces apoptosis in HSC-39 cells. HSC-39 ( $5 \times 10^5$  cells/ml) cells were cultured in medium (A), TGF-β1- (1.5 ng/ml) containing medium (B), the supernatant from GT38 cells (C), anti-TGF-β1 Ab- (1 μg/ml) treated TGF-β1 (1.5 ng/ml) containing medium (D) or anti-TGF-β1 Ab- (1 μg/ml) treated supernatant (E) for 48 h. The cells were fixed with 70% ethanol, stained with propidium iodide, and analyzed by flow cytometry. The DNA content of the non-gated population was expressed as the histogram of propidium iodide fluorescence intensity. The percentage of apoptotic cells (<G<sub>1</sub>; defined by horizontal bars) is denoted in each histogram.

and GT39 cells up-regulated the expressions of PAI-1 and junB in HSC-39 cells (data not shown), as demonstrated by TGF-β1 in Fig. 4.

## DISCUSSION

We have provided new evidence that EBV-infected epithelial cell lines GT38 and GT39 are resistant to TGF-β1 and yet produce biologically active TGF-β1.

First, we demonstrated that GT38 and GT39 cells, in contrast to HSC-39 cells (Yanagihara and Tsumuraya, 1992), are resistant to TGF-β1-mediated growth inhibition and apoptosis. There are a number of potential mechanisms for this lack of TGF-β responsiveness, i.e., mutational inactivation or deletion of TGF-β receptors or SMAD proteins which are signaling molecules for TGF-β1 (Heldin *et al.*, 1997; Massague, 1998). Lost sensitivity to TGF-β is common in several kinds of carcinoma. Changes in expression of the TGF-β R II have been found in gastric (Park *et al.*, 1994), colorectal

(Markowitz *et al.*, 1995; Wang *et al.*, 1995), and breast (Sun *et al.*, 1994) carcinomas due to genetic changes in TGF-β R II. Escape from TGF-β sensitivity has been reported to result from inactivation of the TGF-β R I gene in a gastric carcinoma cell line (Kim *et al.*, 1996). Decreased expression of TGF-β Rs I and II have been observed in gastric and thyroid carcinomas (Ito *et al.*, 1992; Lazzereschi *et al.*, 1997). We demonstrated the expression of mRNAs and proteins of TGF-β Rs I and II in GT38 and GT39 cells (Fig. 2). These results suggest that the resistance of GT38 and GT39 cells to TGF-β1-mediated growth inhibition and apoptosis is not due to the loss of TGF-β Rs.

Second, we found that TGF-β1 induced EBV reactivation in GT38 and GT39 cells that were derived from gastric tissues. TGF-β induces the viral productive cycle in Burkitt lymphoma cell lines and marmoset B lymphocytes immortalized with EBV (di Renzo *et al.*, 1994; Wittmann *et al.*, 1982). GT38 and GT39 cells had about 1% EA-positive cells before treatment with TGF-β1 and the EA-positive cells increased to about 8% at 72 h after TGF-β1 treatment (data not shown). The level of EBV reactivation with TGF-β1 was similar to that with 12-O-tetradecanoylphorbol-13-acetate (TPA), which also induced EBV reactivation in these cells (Fig. 3; Kanamori *et al.*, 2000). These results indicated that EBV was reactivated in part of GT38 and GT39 cells by TGF-β1, although these cells were resistant to TGF-β1-mediated growth inhibition and apoptosis (Figs. 1, 3).

Third, we analyzed the TGF-β1 signaling pathways in HSC-39 and GT38 cells, finding different activation patterns of the TGF-β1-mediated signaling molecules. TGF-β1 clearly induced PAI-1 and junB expression in HSC-39 cells; however, it induced junB, but little PAI-1 in GT38 cells (Fig. 4). These results suggest that EBV in GT38 and GT39 cells is likely to be reactivated via the junB pathway induced by TGF-β1.

Fourth, we detected TGF-β1 mRNA in GT38 and GT39 cells and the biologically active TGF-β1 in the supernatants (Figs. 5–7; Table 1). Using a specific antibody to TGF-β1, we showed clearly that a part of growth inhibition and induction of apoptosis in HSC-39 cells were due to TGF-β1 in the supernatants from GT38 and GT39 cells. The TGF-β1 level in the supernatants from GT38 cells inhibited the growth of HSC-39 cells, but not the growth of GT38 cells (Table 1; Fig. 6). These data were consistent with the result that GT38 and GT39 cells are resistant to the exogenous TGF-β1-mediated growth inhibition and apoptosis (Fig. 1).

TGF-β1 produced by GT38 or GT39 cells does not inhibit the growth of those cells but can inhibit growth of other TGF-β1-susceptible cells. One can hypothesize that EBV-infected cells have resistance to TGF-β1-mediated growth inhibition, and therefore can selectively outgrow in the neighboring cells which are susceptible for TGF-β1 produced by EBV-infected cells. Such EBV-in-

ected cells are selected and can expand the clonal EBV-infected cells as observed in EBV-associated gastric carcinoma (Imai *et al.*, 1994). However, we do not have the direct evidence that the resistance of GT38 and GT39 cells to TGF- $\beta$ 1 is due to by EBV infection itself. Furthermore, the EBV latency of type in these cell lines (Tajima *et al.*, 1998; Takasaka *et al.*, 1998; Murakami *et al.*, 2000) does not fit in the latency I observed in most EBV-associated gastric carcinomas (Imai *et al.*, 1994, Sugiura *et al.*, 1996). It remains to be studied that EBV infection induces the loss of TGF- $\beta$  responsiveness in epithelial cells.

Induction of TGF- $\beta$ 1 has been proposed to play a role in the immunosuppression observed during human T-lymphotropic virus type 1 and cytomegalovirus infections (Kim *et al.*, 1990; Michelson *et al.*, 1994). Induction of TGF- $\beta$ 1 could be a common mechanism used by human viruses to down-regulate host immune responses.

In summary, we have demonstrated that the human gastric tissue-derived epithelial cell lines GT38 and GT39 are resistant to the growth inhibition and apoptosis but are sensitive to EBV reactivation by TGF- $\beta$ 1. They produce TGF- $\beta$ 1, which inhibits TGF- $\beta$ 1-sensitive cells. To explore the role of EBV infection in the development of EBV-associated gastric carcinoma, we have presented a model for EBV infection involving the resistance to TGF- $\beta$ 1-mediated growth inhibition.

## MATERIALS AND METHODS

### Cell lines and medium

GT38 and GT39 cells were EBV-positive epithelial cell lines from human gastric tissues (Tajima *et al.*, 1998). The HSC-39 cell line was derived from a human signet ring cell gastric carcinoma (Yanagihara *et al.*, 1991). The MKN1 and NUGC3 cell lines derived from gastric carcinomas were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air.

### Antibodies and other reagents

TGF- $\beta$ 1 was purchased from Pepro Tech EC Ltd. (London, U.K.). Anti-ZEBRA mouse monoclonal Ab was developed in our laboratory. Anti-early antigen (EA)-D mouse monoclonal Ab was purchased from DuPont (Wilmington, DE). Anti-human TGF- $\beta$ 1 Ab was purchased from Genzyme/Techne (Cambridge, MA) and from R&D Systems, Inc. (Minneapolis, MN). Biotinylated anti-human TGF- $\beta$ 1 Ab were purchased from R&D Systems. Anti-TGF- $\beta$  R I (V-22) and anti-TGF- $\beta$  R II (H-567) Abs were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The human cDNA probes for TGF- $\beta$ 1, TGF- $\beta$  Rs I and II

were kindly provided by Drs. R. Derynk and K. Miyazono, respectively. cDNAs for junB and PAI-1 were provided by Drs. S. Sharma and D. J. Loskutoff, respectively.

### Colorimetric assay for cell proliferation

The cell growth was assayed by the incorporation of MTT dye (Chemi-Con Int. Inc., Temecula, CA). Cells were seeded in 96-well plates (Corning Inc., Corning, NY) at  $5 \times 10^3$  cells/100  $\mu$ l per well for 24 h and then cells were cultured with various concentrations of TGF- $\beta$ 1 for 72 h. Ten microliters of MTT solution (5 mg/ml) was added to each well, and the plates were incubated for 4 h at 37°C. One hundred microliters of isopropanol with 0.04 N HCl was added to each well and mixed by repeated pipetting. Absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 655 nm with a microplate reader (Model 550, Bio-Rad, Richmond, CA).

### DNA fragmentation assay

Cells were lysed in an extraction buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% sodium dodecyl sulfate (SDS) and 0.1 mg/ml proteinase K) at 37°C for 18 h. At the end of the incubation period, the mixture was treated with an equal volume of phenol-chloroform-isoamylalcohol (1:1:0.1). DNA was precipitated from the water phase by adding 2.5 vol ethanol and 0.3 M sodium acetate. The precipitated DNA was rinsed once with 70% ethanol, dried under vacuum, and finally solubilized into TE buffer (10 mM Tris-HCl, pH 7.6, and 1 mM EDTA) supplemented with RNase I (Promega, Madison, WI), 0.2 mM sodium acetate, and 50 mM EDTA. Following incubation at 37°C for 1 h, SDS was added to the mixture at a final concentration of 0.1% to inactivate the RNase activity. DNAs were precipitated as described above and finally solubilized into TE buffer. Five micrograms of DNAs were subjected to 2% agarose gel electrophoresis at 100 V for about 1 h. DNA was visualized with ethidium bromide.

### Treatment of TGF- $\beta$ 1

Logarithmically growing cells were suspended to a final concentration of  $1 \times 10^6$  cells/ml with fresh medium. The cells were cultured for the indicated time with various concentrations of TGF- $\beta$ 1.

### Western blotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and treated with 10% trichloroacetic acid on ice for 15 min, collected in a microcentrifuge tube, and centrifuged at 15,000 rpm, 0°C for 5 min. The pellets were lysed in 100  $\mu$ l lithium dodecylsulfate (LDS) sample buffer containing 125 mM Tris-HCl (pH 6.8), 2.3% (w/v) LDS, 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, and 10  $\mu$ g/ml bromophenol blue, and boiled for 5 min.

Proteins were resolved by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA). Blotting of equal amounts of protein to the membrane was confirmed by Coomassie brilliant blue R-250 dye staining. After blocking incubation with PBS-Tween 20 containing 5% nonfat dry milk powder for 2 h at RT, the membranes were incubated with either anti-ZEBRA or anti-EA-D overnight at 4°C. Following primary antibody incubation, the membranes were incubated with anti-mouse antibody (New England Biolabs, Beverly, MA) conjugated to alkaline phosphatase. The membranes were further processed using the Western-light chemiluminescent detection system (Tropix, Bedford, MA).

### Northern blotting

Total RNA was prepared by the guanidium thiocyanate method per instructions (Isogen, Wako, Osaka, Japan) and Northern blots were performed as previously described (Gao *et al.*, 1999). Briefly, each RNA sample was denatured for 5 min at 65°C in 50% formamide and 2.2 M formaldehyde, and then electrophoretically separated in 1% agarose gels. The RNA was then transferred onto Hybond-N filter paper (Amersham, Tokyo, Japan) and prehybridization was performed for 24 h at 42°C in 50% formaldehyde, 5× SSC, 2.5× Denhardt's solution, 0.1% SDS, and 300 µg salmon sperm DNA/ml. The membranes were hybridized 24 h at 42°C with random primer-labeled cDNA probes, respectively. After hybridization, the membranes were washed twice at 65°C in 1× SSC, 0.1% SDS for 10 min and were exposed to Kodak XAR film. Equal loadings of RNA were confirmed by hybridization with glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probe.

### Assay of TGF-β1 by ELISA

TGF-β1 was quantified by the fluorometric sandwich ELISA described previously (Sakaguchi *et al.*, 1989). F96 Maxisorp Nunc-Immuno Plate (Nalge Nunc International, Dansk Kraftemballage, Denmark) was coated with 4 µg/ml of anti-human TGF-β1 Ab (R&D Systems) in 0.05 M carbonate-bicarbonate buffer (pH 9.5) for 3 h at 37°C. The plate was emptied and then coated with 1% BSA-PBS at 37°C for 1 h. After washing, supernatants and several concentrations of TGF-β1 in 1% BSA-PBS Tween 20 were added to the wells, and the plate was incubated overnight at 4°C. The plate was washed, and 100 ng/ml biotinylated anti-human TGF-β1 Ab (R&D Systems) was added as a detector antibody. The plate was incubated for 1 h at room temperature. After washing, β-D-galactosidase-conjugated streptavidin (Gibco-BRL, Gaithersburg, MD) diluted 10,000 was added. The plate was incubated for 1 h at room temperature. After washing, 0.1 mM 4-methylumbelliferyl-β-D-galactoside (Sigma, Steinheim, Germany) in 0.1 M NaCl, 1 mM MgCl<sub>2</sub>, 0.1% BSA, 10 mM Na-phosphate, and 0.02% NaN<sub>3</sub> was added to each

well. The plate was incubated for 2 h at 37°C. The enzyme reaction was stopped with 0.1 M glycine-NaOH (pH 10.2), and the fluorescence intensity was read as fluorescence units on a microplate fluorescence reader (Cyto Fluor, PerSpective Biosystems, Osaka, Japan).

### Assay of biological active TGF-β1

Cells were seeded in complete medium at  $1 \times 10^6$  cells/ml and incubated for 24 h. The supernatants were collected from cultures after centrifugation, filtered through a 0.45-µm filter, and used immediately for the growth inhibition test on HSC-39 or GT38 cells. Cells were seeded in 96-well plates at a density of  $5 \times 10^3$  cells/well with supernatant from GT38 or GT39 cells. After 72 h, cell growth was measured by MTT assay.

### Neutralization test of TGF-β1 with anti-TGF-β1 antibody

Neutralizing activity of TGF-β1 with anti-human TGF-β1 antibody was measured as abrogation of growth inhibition to HSC-39 cells by TGF-β1 in the supernatants from GT38 or GT39 cells. Various concentrations of the anti-human TGF-β1 antibody were mixed with the supernatants for 10 min at 37°C, which were then added to HSC-39 cells. The cells were cultured for 72 h at 37°C and cell growth was measured using the MTT assay.

### Fluorescence-activated cell sorting analysis for apoptosis

Cells were washed in cold PBS, and single cell suspensions were fixed with 70% ethanol at 4°C for 1 h, and then incubated with PBS solution containing 50 µg/ml RNase A at 37°C for 20 min. DNA content of the cells was measured after staining with 50 µg/ml propidium iodide by flow cytometry using an EPICS XL flow cytometer (Coulter Corp., Miami, FL). Cells containing less DNA than those in G<sub>0</sub>/G<sub>1</sub> phase represent apoptotic cells.

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